ELECTRICAL PROPERTIES AND ULTRASTRUCTURE OF *MYCOPLASMA* MEMBRANES

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ABSTRACT *Mycoplasma*, in particular species *laidlawii* and *gallisepticum*, are found to have a very small, low frequency conductivity as would be predicted by the dielectric model for bacteria and their apparent lack of cell wall structure. Membrane capacitance values for the two organisms are both about 0.9 $\mu F/cm^2$, although electron micrographs show that the membrane of *M. gallisepticum* is 20–40 A thicker than that of *M. laidlawii*.

INTRODUCTION

The *Mycoplasma* are a genus of small free-living cells. Evidence from electron microscopy indicates that they are characterized by the absence of any identifiable cell wall structure external to the plasma membrane, and chemical analysis has confirmed the absence of bacterial cell wall components, such as mucopeptide polymer (Razin, 1969). If this is true, the *Mycoplasma* should have electrical properties similar to those of bacterial protoplasts. In particular, the dielectric model for the bacterial cell predicts that these cells should have very small effective conductivities at low frequencies (Carstensen et al., 1965; Carstensen, 1967; Carstensen and Marquis, 1968; Einolf and Carstensen, 1969).

Mycoplasma laidlawii and *Mycoplasma gallisepticum*, as will be reported here, do have low conductivities. Furthermore, membrane capacities for both organisms are about 0.9×10^{-2} farads/m^2 (0.9 $\mu F/cm^2$).

MATERIALS AND METHODS

Mycoplasma

The organisms used in this study, obtained from Professor M. E. Tourtellotte (University of Connecticut, Storrs, Conn.), were *M. laidlawii* B and *M. gallisepticum* strain A5969.

Media and Growth

The cells were grown in static cultures, at 37°C, in a tryptose broth medium (containing 1% glucose and 1% pleuropneumonia-like organism [PPLO] serum fraction), as previously described (Maniloff, 1969). To obtain exponentially growing cells, 2-liter cultures (started with a 20 ml inoculum) were harvested at 16–20 hr. An aliquot was assayed using blood agar plates. In all cases, the titer was in the range of 1–5 $\times$ 10^8 clone-forming units/ml, which for these cells and media confirmed that the cultures were in the exponential growth phase (Maniloff, 1969).

Dielectric Measurements

The cells were collected by continuous flow centrifugation (Ivan Sorvall, Inc., Norwalk, Conn.) and washed three times in 0.2 molal sucrose–0.1 M NaCl. Dielectric measurements used to determine membrane capacitance were made with the cells in this medium. Then, to obtain estimates of the effective conductivity of the cells, they were washed four or more times in 0.2 molal sucrose to reduce the conductivity of the environment.

Dielectric measurements in the frequency range 1–200 MHz were made on suspensions of cells using a Hewlett-Packard Model 250A RX meter (Hewlett-Packard Co., Harrison Div., Berkeley Heights, N. J.). Conductivity measurements at 1592 Hz were made with a Wayne Kerr Model B221 Universal Bridge (Wayne Kerr Corp., Montclair, N. J.). Techniques for these measurements have been described previously (Carstensen et al., 1965; Pauly and Schwan, 1966).

The effective complex conductivity σ_2^+ of the cells was determined from the relation (Fricke, 1955)

$$\frac{\sigma^+ - \sigma_1^+}{\sigma^+ + 2\sigma_1^+} = p\,\frac{\sigma_2^+ - \sigma_1^+}{\sigma_2^+ + 2\sigma_1^+}, \tag{1}$$

where the complex conductivity $\sigma^+ = \sigma + j\omega\epsilon_0\kappa$, σ being the real conductivity, ω the angular frequency, ϵ_0 the permittivity of free space, and κ the relative dielectric constant; σ^+ and σ_1^+ are the measured complex conductivities of suspension and suspending medium respectively and p is the volume fraction of the suspension occupied by the cells. For accuracy, values of p in the range 0.2–0.5 were used in the measurements. When the environmental conductivity is high, i.e. $\sigma_1 \gg \sigma_2$, the real part of equation 1 at low frequencies reduces to

$$p = \frac{\sigma_1 - \sigma}{\sigma_1 + \sigma/2}. \tag{2}$$

Hence the low frequency conductivity measurements can provide a measure of p for the analysis of the membrane capacitance data. As $\sigma_1 \rightarrow \sigma_2$, calculations of σ_2 from measured values of σ, σ_1, and p become less sensitive to p (Einolf and Carstensen, 1967). Thus, for estimates of σ_2 at low ionic strengths, a sufficiently accurate determination of p can be obtained from the size of the pellet of packed cells after centrifuging the sample (a kind of hematocrit determination). For measurements at high ionic strength it is found that these cells pack so that approximately 70% of the pellet is cell volume. Even without knowledge of cell volume it was possible to set upper and lower limits on the effective conductivity of the cells by measuring them in environments with σ_1 first greater than and then less than σ_2.

Individual cell volume and hence radius of *M. laidlawii* was determined by measuring the volume fraction p of cells in a suspension as indicated above and dividing by a cell count

obtained by using a Petrov-Hauser counting chamber (G. A. Hauser & Son, Philadelphia, Pa.) and phase-contrast microscope. The radius of *M. gallisepticum* was determined by direct measurements on phase-contrast photomicrographs of cells in aqueous suspension. These radii were in agreement with values determined by electron microscopy.

Capacitance Calculations

Fricke's (1955) theory for the dielectric properties of suspensions of shelled spheres suggests a simple, yet rigorous, two-step method for calculation of the membrane capacitance. First, the effective, homogeneous complex conductivity σ_2^+ of the cell is computed from equation 1 as described. This requires a knowledge of the complex conductivities σ^+ and σ_1^+ of the suspension and suspending fluid respectively, and the volume fraction p, all of which are directly measureable. Thus no assumptions or simplifications in the theory are necessary to compute σ_2^+. Error analysis for measurements of this kind have been discussed previously (Carstensen and Smearing, 1967). Second, the complex conductivity σ_m^+ of the outer shell (the membrane) of the suspended particle is calculated from its effective conductivity σ_2^+ (Fricke, 1955)

$$\frac{\sigma_i^+ - \sigma_m^+}{\sigma_2^+ + 2\sigma_m^+} = \left(\frac{a_i}{a}\right)^3 \frac{\sigma_i^+ - \sigma_m^+}{\sigma_i^+ + 2\sigma_m^+}, \tag{3}$$

where σ_i^+ is the complex conductivity of the cytoplasm, a is the over-all radius of the cell, and a_i is the radius of the cytoplasmic core, i.e., $a - a_i = t$ is the thickness of the membrane.

TABLE I

DIELECTRIC AND STRUCTURAL PARAMETERS OF TWO *MYCOPLASMA*

Parameters	*M. laidlawii*	*M. gallisepticum*
Cell diameter, mean ±SE, μm	0.96 ±0.06	0.53 ±0.02
Membrane thickness, *A*	70–80	110–120
Peak-to-peak membrane distance, *A*	50–60	70–80
No. of samples	12	3
Effective homogeneous conductivity, σ_2 (1592 Hz) mean ±SE, *mho/m*	0.004 ±0.001	0.002 ±0.001
Effective homogeneous dielectric constant κ_2 (1 MHz)	470 ±20	260 ±7
Membrane capacitance, C_m, mean ±SE, *farads/m²* × 10^2	0.89 ±0.04	0.92 ±0.03
Surface charge density, *coul/m²*	−0.01	−0.01
Predicted σ_2 (equation 4), *mho/m*	0.002	0.004

Values of the effective, homogeneous dielectric constant κ_2 and conductivity σ_2 were calculated from observed data using equation 1.

Membrane capacitance C_m is calculated from low frequency limits of κ_2 using equation 3.

In the measurements, volume concentrations of the cells in suspension ranged from 0.2 to 0.5.

Environmental conductivities σ_1 in the range 0.001–0.01 mho/m were used in the measurements leading to σ_2.

In the measurements for κ_2, environmental conductivities were of the order of 1 mho/m.

Of these quantities, a is directly measureable, σ_2^+ is calculated as described above, values of σ_i^+ can be estimated from dielectric measurements at high frequencies, and t can be measured by electron microscopy as described below. The results are insensitive to the choice of σ_i^+ as long as $\sigma_i^+ \gg \sigma_m^+$ which is the case for these cells. In fact, in the calculation of the membrane capacitance $C_m = \epsilon_0 \kappa_m / t$, even the value of t used in the calculations is not critical. As an example, with *M. gallisepticum* a decrease by a factor of two in the assumed value of membrane thickness would result in a 2% decrease in the value of membrane capacitance. The effects of the membrane on σ_2^+ are greatest at low frequency. Hence low frequency limits of κ_2 are used in the capacitance calculations. For membrane capacitance calculations the following values were used (See Table I): cytoplasmic conductivity $\sigma_i = 0.5$ mho/m, cytoplasmic dielectric constant $\kappa_i = 100$, and membrane thickness $t = 100$ A. All calculations were carried out numerically using equations 1 and 3 directly without approximations or any assumptions other than those specifically stated. Not only is this procedure simple and direct, but for cells as small as *M. gallisepticum* this method is preferable to the use of approximate relationships which assume that the membrane has negligible thickness by comparison with the radius of the cell.

The relaxation frequency for *M. laidlawii* is approximately 15 MHz; for *M. gallisepticum*, about 30 MHz. Thus the 1 MHz values of κ_2 are very close to the low frequency limits of the effective dielectric constants for the cells.

Surface Charge Density

The surface charge density was measured using a microelectrophoresis method, described elsewhere (Einolf and Carstensen, 1967). The contribution of the surface charge Σ to the effective, homogeneous conductivity of the particle is (Fricke and Curtis, 1936)

$$\sigma_2 = \frac{2e_0 \Sigma u}{a}, \qquad (4)$$

where e_0 is the electronic charge, u is the mobility of the counterion, and a is the radius of the particle. In the calculations, the mobility of the counterions was taken to be equal to that of a sodium ion in 0.2 M sucrose, i.e., $u = 3 \times 10^{11}$ m^2/coul·v·sec. This takes into account the effect of sucrose on the mobility but does not consider possible effects which may arise from electrostatic interaction between the counterions and fixed charges on the surface of the cell (Schwartz, 1962). Used in this way, equation 4 should give rough estimates of the contribution which surface conductance will make to the effective, homogeneous conductivity of the cells.

Electron Microscopy

For electron microscopy, pellets of the cells used in the electrical measurements were fixed in both glutaraldehyde and osmium tetroxide, embedded in Epon, sectioned, stained with uranyl acetate, and examined with a Siemens Elmiskop I (modified to IA) (Siemens Corp., Iselin, N. J.) as previously described (Maniloff et al., 1965). For membrane measurements, the microscope was calibrated with a carbon grating replica.

Membrane thickness measurements were made using a calibrated Bausch & Lomb measuring magnifier (Bausch & Lomb, Inc., Rochester, N. Y.). For peak-to-peak distances, micrographs (taken at × 54,000) were enlarged 3 times onto Kodak Projector Slide Plates (Eastman-Kodak Co., Rochester, N. Y.), and the plates were scanned in a Beckman Model R-110 Densitometer (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Electron Microscopy

Fig. 1 shows electron micrographs of typical sections of the *M. laidlawii* and *M. gallisepticum* pellets used for the electrical measurements. From these data, the cell diameters were estimated to be 0.9–1.0 μm for *M. laidlawii* and 0.50–0.55 μm for *M. gallisepticum*, in agreement with the values obtained by the methods described above (Table I) and in agreement with previously published values for these organisms (Maniloff et al., 1965; Maniloff, 1970). Since we have noted similar sizes for washed and unwashed cells, the washings involved in these studies do not appear to have caused a measurable change in cellular volume although the cells have become more spherical in shape. The intracellular material is not well preserved in the washed cells (Fig. 1).

The cells are bounded by a unit membrane which has a thickness of 70–80 A for *M. laidlawii* and 110–120 A for *M. gallisepticum*. This is in agreement with other measurements on the membranes of *M. laidlawii* by Razin et al. (1965) and Maniloff (1970), and of *M. gallisepticum*, by Maniloff et al. (1965). Peak-to-peak distances were measured from densitometer scans of the micrographs (Fig. 2) and were 50–60 A for *M. laidlawii* and 70–80 A for *M. gallisepticum*. Shoulders on the peaks precluded measurements of the peak half-heights as a measure of membrane thickness. (It should be noted that these shoulders have been lumped subjectively into the over-all membrane thicknesses, since one tends to measure optically between the densest edges of the membrane.) The shoulders on the inside clearly seem to be absorbed cytoplasmic material. On the external membrane surface they are probably due to extracellular material absorbed from the medium.

From both membrane thickness and peak-to-peak distance measurements, it is concluded that there is a 20–40 A difference in the membrane thickness of *M. laidlawii* and *M. gallisepticum*. Since the fixing procedures for the two *Mycoplasma* were identical, this difference seems to be significant. (Measurements of the electron micrographs themselves involve errors of roughly 5–10%.) This difference will be discussed further below, in terms of the electrical studies.

Dielectric Data

Dielectric studies were performed on 12 separate samples of *M. laidlawii* and three samples of *M. gallisepticum*. With cells in sucrose plus 0.1 M NaCl, measurements were carried out at 1592 Hz and throughout the frequency range 1–200 MHz. After washing in sucrose, measurements were made only at 1592 Hz. The data display the expected β-dispersion (Schwan, 1957), with high frequency limits which show cytoplasmic conductivities σ_i to be of the order of 0.5 mho/m. Values of the low frequency effective, homogeneous conductivities, dielectric constants calculated with equation 1, and membrane capacitance calculated with equation 3 are summarized in Table I. Cells for each of the samples indicated were grown on different

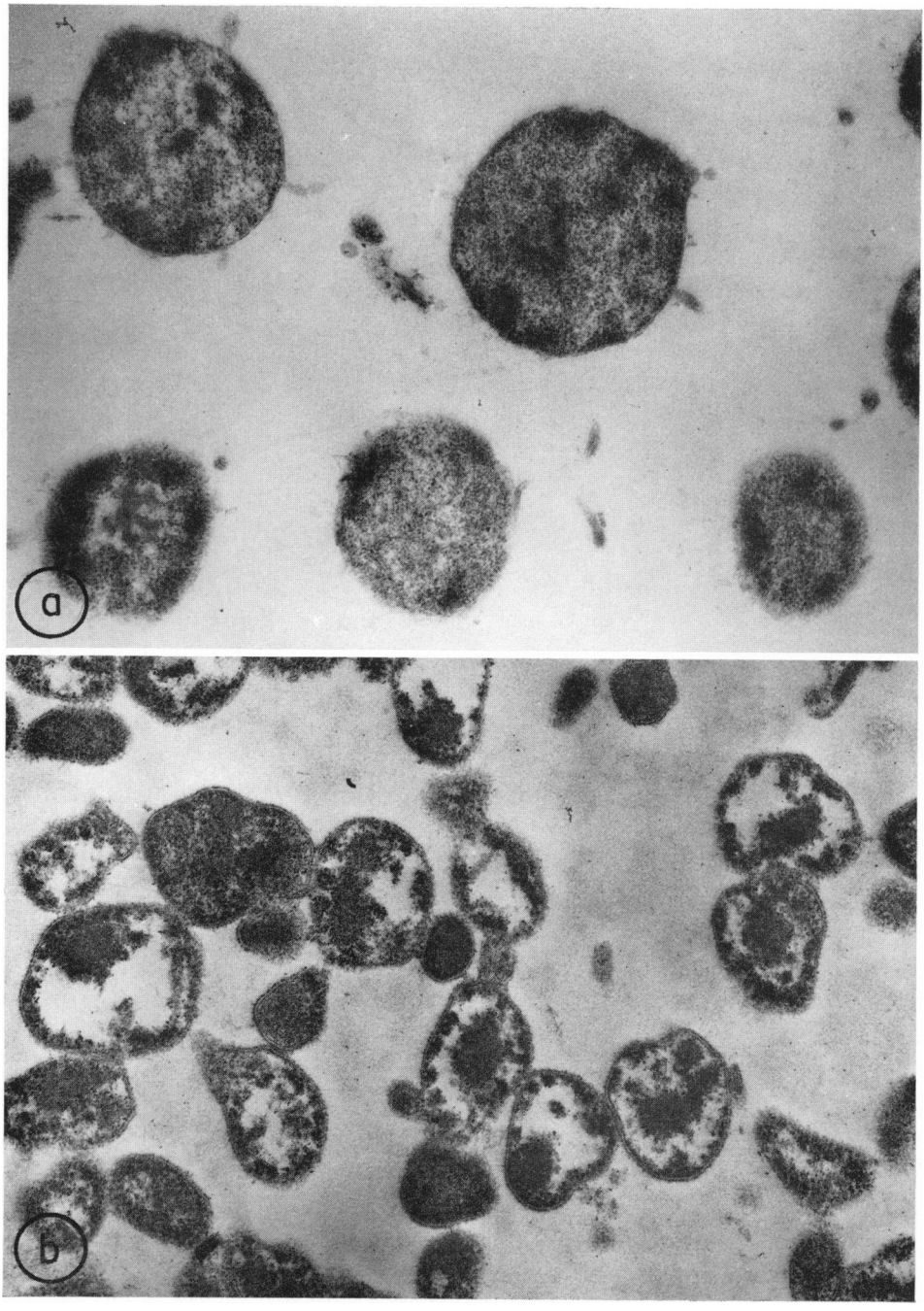

FIGURE 1 Electron micrographs of cells used for electrical measurements. (*a*) *M. laidlawii* (*b*) *M. gallisepticum*.

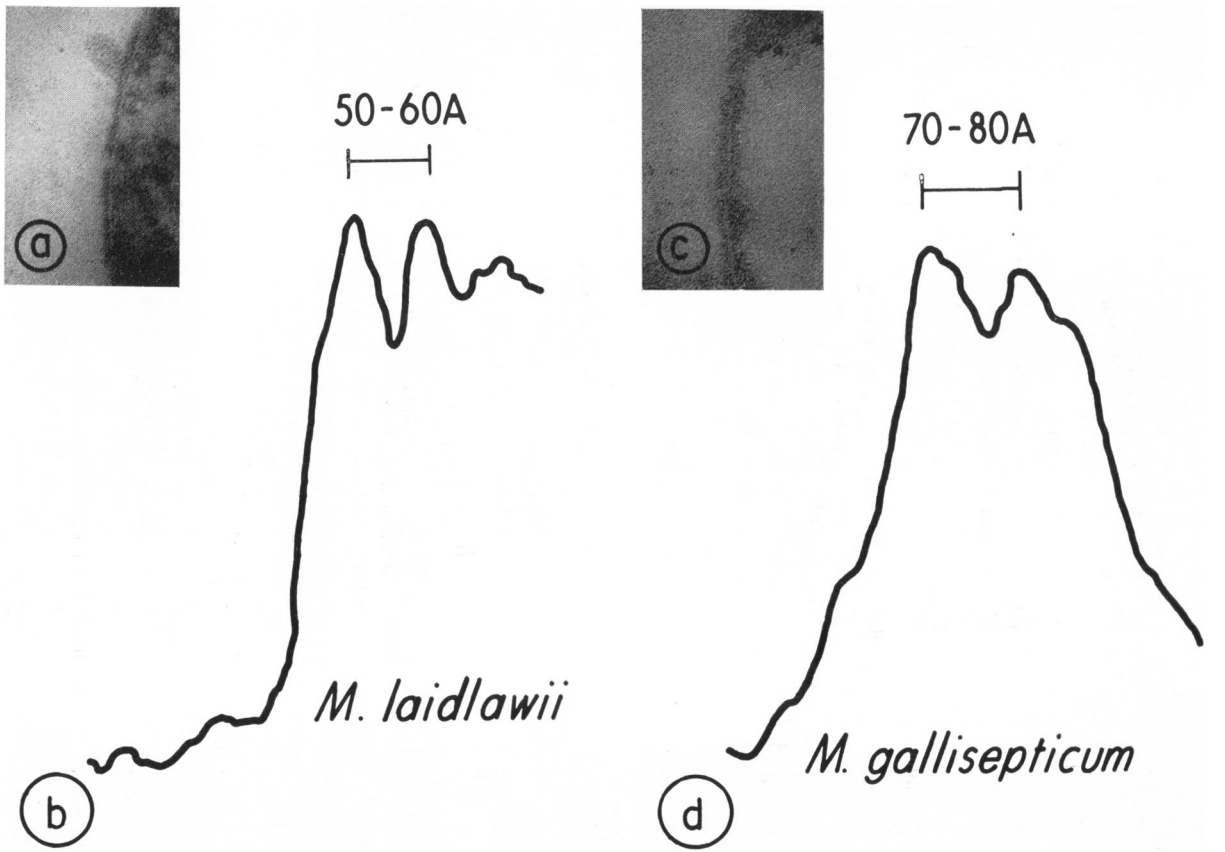

FIGURE 2 Electron micrographs of *Mycoplasma* membranes. (*a*) *M. laidlawii* (*c*) *M. gallisepticum*. Corresponding densitometer traces are shown in (*b*) and (*d*).

days but under identical culture conditions and methods of preparation. In spite of the care taken to ensure uniformity, the sample-to-sample variation in conductivity for *M. laidlawii* was rather large.[1] Because of the large number of samples studied, however, the average values can be regarded as meaningful. The reproducibility with *M. gallisepticum* was much better.

DISCUSSION

Conductivity

From the dielectric point of view, the typical bacterial cell is a three-phase structure consisting of a conducting core (the cytoplasm), contained within a thin insulating membrane, which in turn is surrounded by a porous, conducting cell wall. At low frequencies, the effective, homogeneous conductivities of bacterial cells depend upon the properties of the cell wall. Organisms with morphologically distinct walls have low frequency conductivities ranging from 0.02 to 0.2 mho/m (Einolf and Carstensen, 1967). Enzymatically removing the cell walls of *Micrococcus lysodeikticus* reduces the low frequency conductivity from 0.1 to 0.001 mho/m (Einolf and Carstensen, 1969). After removal of the cell wall structure, the magnitude of the effective conductivity can be explained by the surface conductance which is related to the fixed charge on the surface of the protoplast. Except for the very thin shoulder on the outer surface (Fig. 2), there is no evidence in the electron micrographs of a surface structure external to the membrane of the *Mycoplasma*. From this observation one would predict that the low frequency conductivity of these organisms would be very small—perhaps comparable to the protoplasts of other bacteria.

The data of Table I are consistent with the absence of extracellular surface layers in the *Mycoplasma*. To obtain a rough estimate of the values of σ_2 which would be expected from surface conductance alone, microelectrophoresis measurements were performed. Electrophoretic mobilities and hence surface charge densities were found to be the same for the two organisms. When the corresponding calculated effective conductivities are compared with the observed values (Table I), we see that the conductivities found for *M. gallisepticum* are well within upper limits which would be set by surface conductance. The conductivity of *M. laidlawii* appears to be a little high, but in view of the assumptions involved in the theory, the observed values are probably not significantly different from those predicted from surface conductance. Yet the possibility of either a thin conducting structure external to the membrane or a finite membrane resistance for this organism must be admitted. Of these two possibilities the latter is least likely. Membrane resistance values of the order of 10^{-4} ohms m^2 (1 ohm cm^2) would be required to explain effective con-

[1] In studies of older *M. laidlawii* cultures (72–144 hr), conductivities of two out of five samples had effective conductivities in excess of 0.02 mho/m. At this age the cultures were no longer viable (Maniloff, 1969).

ductivities as high as 0.004 mho/m observed for *M. laidlawii*. This is a very low resistance for a normal cytoplasmic membrane. Where membrane resistances as low as 10^{-4} ohm m^2 have been observed, the cells are readily permeable to sucrose (Carstensen and Smearing, 1967; Carstensen et al., 1969). Yet the *Mycoplasma* were stabilized osmotically for these experiments with 0.2 molal sucrose.

Capacitance

As shown in Table I the membrane capacitance values for the two organisms investigated here are approximately 0.9×10^{-2} farads/m^2. These may be compared to values of roughly 0.85×10^{-2} for erythrocytes (Fricke, 1953) and 1.0×10^{-2} farads/m^2 for protoplasts of *Micrococcus lysodeikticus* (Einolf and Carstensen, 1969). The dielectric constant data for *M. gallisepticum* in Table I are in excellent agreement with those reported by Schwan and Morowitz (1962) for the same organism. Their mean, effective, homogeneous dielectric constant for three experiments with these cells was 250 as compared with 260 in the present study. More recently Maniloff et al. (1965) have shown that *M. gallisepticum* is somewhat larger than was originally assumed in the calculations of membrane capacitance (Schwan and Morowitz, 1962). When the revised size data are used, the membrane capacitance values calculated from the Schwan-Morowitz data agree very closely with those given in Table I.

The similarity in the capacitances of *M. laidlawii* and *M. gallisepticum* suggests that both cells are surrounded by an insulating barrier of about the same thickness. In contrast, the electron microscopic observations show that the membrane thicknesses of these cells are significantly different. Therefore, the difference in the membrane thickness, as measured by thin-section electron microscopy, probably reflects membrane ultrastructure other than the insulating dielectric layer. In other words, it is possible that the insulating barrier which surrounds the cell is only one layer in the laminated structure which is usually identified as the "cytoplasmic membrane".

CONCLUSION

From the dielectric point of view the *Mycoplasma* appear to have a conducting cytoplasm surrounded by an insulating membrane with a capacitance about 0.9×10^{-2} farads/m^2. There is no dielectric evidence of a cell wall structure in *M. gallisepticum*. With a slight reservation the same statement can be made for *M. laidlawii*.

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